

EXHIBIT E

Simvastatin Attenuates Trinitrobenzene Sulfonic Acid-Induced Colitis, but Not Oxazalone-Induced Colitis

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Abstract *Purpose* To determine whether simvastatin is able to inhibit inflammation in trinitrobenzene sulfonic acid (TNBS)-induced or oxazalone (OXA)-induced colitis. *Results* In the prophylactic protocol, simvastatin dose-dependently suppressed the decrease in body weight and inflammatory grade of TNBS-treated mice. In contrast, in the therapeutic protocol, no significant difference in body weight reduction was observed between simvastatin-treated and control mice. IFN- γ release from LP cells was significantly suppressed in mice receiving high-dose simvastatin in the prophylactic protocol. In contrast to TNBS colitis, even high-dose prophylactic simvastatin had no suppressive effects on either weight reduction or the inflammatory grade in OXA colitis. *Conclusion* Our results indicate that simvastatin negatively regulates inflammation in TNBS-induced colitis, but not in OXA-induced colitis. In TNBS-induced colitis, simvastatin suppressed the Th1-polarized immune response. Our findings suggest that simvastatin has potential effects as a therapeutic agent in human inflammatory bowel disease, particularly Crohn's disease.

Keywords Simvastatin · Statin · TNBS · Colitis

Introduction

Statins are known to be 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors and are widely used to reduce cholesterol in patients with hyperlipidemia leading to cardiovascular morbidity [1]. Recent clinical studies have suggested that statins may also have immunosuppressive effects and be useful in the treatment of inflammatory diseases. For example, clinical trials have demonstrated that simvastatin and pravastatin have an effect on the prevention of transplant rejection after heart transplantation [2, 3]. Statins also reduced highly sensitive C-reactive protein levels [4, 5] and vascular proinflammatory cytokines, such as TNF- α , IL-1, and IL-6 [6] in hyperlipidemia patients.

Although the molecular mechanisms of such anti-inflammatory activities remain unclear, recent studies have provided some insight. Certain statins are able to inhibit the interaction between lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) [7]. Moreover, statins also prevented IFN- γ inducible MHC class 2 expression in non-professional APC [8]. In vivo, using murine models, Youssef et al. [9] demonstrated that atorvastatin rescued experimental Th1 encephalomyelitis, inhibiting the Th1-mediated immunoreponse. Leung et al. [10] also reported an anti-inflammatory role for simvastatin in experimental Th1 arthritis. Recently, it was reported that simvastatin reduced the expected number and volume of new gadolinium-enhancing lesions in relapsing-remitting multiple sclerosis patients in a small open label trial [11]. Atorvastatin has also been reported to decrease the disease score and vascular risk factors in patients with rheumatoid arthritis in a double-blind placebo controlled trial [12].

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Based on these results, statins appear to be beneficial for other Th1-mediated autoimmune diseases. Human inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), are believed to be autoimmune conditions resulting from an abnormal mucosal T cell response to bacteria in the lumen. In CD, mucosal T cells exhibit Th1 phenotype, whereas UC is believed to have Th2 phenotype, but this has not been clearly demonstrated. Three recent articles have shown that pravastatin [13], rosuvastatin [14], and simvastatin [15] are able to reduce disease activity and colonic inflammation in dextran-sulfate (DSS)-induced colitis in mice.

Trinitrobenzene sulfonic acid (TNBS)-induced colitis is a colitis model that results in transmural infiltration and an IL-12-driven, Th1-mediated response [16, 17]. In contrast, oxazalone (OXA)-induced colitis is caused by a polarized Th2-mediated response [16]. We can thus utilize both TNBS-induced and OXA-induced murine experimental colitis as models of human IBD in order to test whether statins are able to suppress such colitis. The present study was conducted in order to elucidate whether statins can inhibit the inflammation of TNBS-induced or OXA-induced colitis.

Methods

Animals

All experiments were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University. Six- to 8-week-old SJL/J male mice were obtained from SLC Inc. (Shizuoka, Japan).

Experimental colitis models

Colitis was induced in SJL/J mice as described previously [18, 19]. Briefly, in order to presensitize mice, abdominal skin was shaved and 150 μ l of either TNBS (2.5% in 50% ethanol) or OXA (3% in 100% ethanol) was applied. Five days after presensitization, mice were intrarectally administered 150 μ l of a solution of 1.5% TNBS or 1% OXA dissolved in 0.9% NaCl and mixed with an equal volume of ethanol. Control mice (CO) were given ethanol in place of TNBS.

Treatment protocols

For the prophylactic protocol, mice were administered 10 or 40 mg/kg i.p. simvastatin daily from 5 days before to 7 days after rectal administration of TNBS or OXA. For the therapeutic protocol, mice were administered 10 or

40 mg/kg i.p. simvastatin daily for a total 7 days after rectal administration of TNBS or OXA. Control mice (NS) for simvastatin treatment received 0.9% NaCl in place of simvastatin.

Histological grading of colitis

Sections of the proximal, middle, and distal colon were fixed in 3% buffered formalin. Tissue sections were subjected to hematoxylin and eosin staining using standard techniques for histological examination. Degree of inflammation was graded semiquantitatively, as described previously [17]. The grading system was as follows: 0, no inflammation; 1 = very low-level inflammation; 2 = low-level inflammation; 3 = high-level inflammation with high vascular density and thickening of the colon wall; 4 = transmural infiltration with loss of goblet cells, high vascular density, and thickening of the colon wall. Grading was performed in blinded fashion.

Isolation of lamina propria (LP) cells

Colons were washed with PBS and were digested with dispase (2 mg/ml, Gibco BRL, Grand Island, NY) at 37°C for 1 h in complete media (RPMI 1640 media supplemented with 4% FCS, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin) in order to remove the epithelial compartment, including intraepithelial lymphocytes. Samples were then minced into small pieces and further digested with collagenase (1 mg/ml, Gibco BRL) and dispase (2 mg/ml) for another hour at 37°C. After washing, cells were purified using the 40%/70% Percoll gradient method to remove epithelial and dead cells. Cells at the interface were isolated and washed with PBS.

Analysis of cytokine production

Colonic LP cells were cultured at 2×10^5 cells in 150 μ l of media (DMEM/F-12 media containing 1% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 250 ng/ml amphotericin, 2 mM L-glutamine, 0.05 mM 2ME, 10 mM HEPES, 1 mM sodium butyrate, and $1 \times$ SITE-3). Cells were either unstimulated or stimulated with anti-TCR- β . Supernatant was collected after 5 days of culture, spun down for cell removal, and frozen at -80°C until cytokine analysis. OptEIA™ cytokine sandwich ELISA kits from PharMingen were used to assess the expression of IL-4, IL-5, IFN- γ , and TNF- α , in accordance with the manufacturer's instructions.

Statistical analysis

All data are expressed as means \pm SEM. Differences between groups were examined for statistical significance using Student's *t*-test or nonparametric Mann-Whitney U test. A *P*-value of less than 0.05 denoted the presence of a statistically significant difference.

Results

Simvastatin prevents mice from developing TNBS colitis

In order to determine whether simvastatin suppresses immune-mediated pathology *in vivo*, we investigated the effects of simvastatin in TNBS-induced colitis (prophylactic protocol). As shown in Fig. 1, SJL/J mice treated with TNBS alone (NS) manifested a severe illness characterized by weight loss and diarrhea peaking on day 4 after intrarectal administration of TNBS. A significant decrease in body weight ($15.1 \pm 1.4\%$; $n = 20$) was seen in TNBS-treated mice in comparison with the CO group (ethanol-treated mice). Simvastatin dose-dependently suppressed the decrease in body weight in TNBS-treated mice. Administration of high-dose (40 mg/day) simvastatin, but not low-dose (10 mg/day) simvastatin, reduced the decrease in body weight ($6.6 \pm 2.0\%$, $n = 19$ vs. $16.4 \pm 1.8\%$, $n = 6$, respectively; $P < 0.01$, high-dose simvastatin in comparison with NS). On macroscopic examination (Fig. 2), while NS colons showed severe

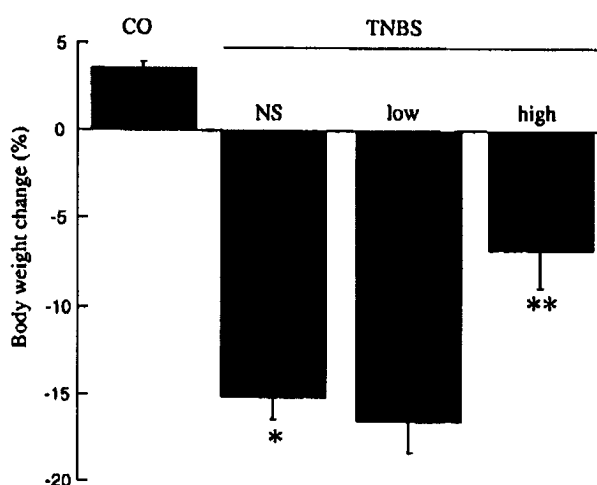


Fig. 1 Prophylactic administration of simvastatin improves the body weight reduction induced by TNBS. A significant decrease in body weight in TNBS-treated mice (NS) was observed. * $P < 0.001$ vs. controls (CO—ethanol-treated mice). Administration (prophylactic protocol) of high-dose (high), but not low-dose (low), simvastatin reduced this decrease in body weight. ** $P < 0.01$ vs. NS

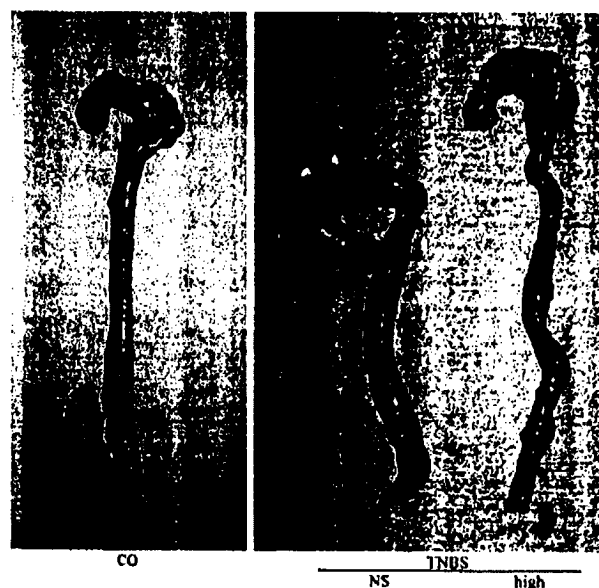


Fig. 2 Prophylactic administration of simvastatin improves the macroscopic lesions induced by TNBS. CO—ethanol-treated mice. NS—TNBS-treated mouse, showing marked edema and shortening of colon length. High—high-dose simvastatin. Note the marked reduction in colitis

colitis involving the entire colon and shortening of colon length, colons from high-dose simvastatin-treated TNBS mice were similar in appearance to those from CO mice. In addition, as shown in Fig. 3a, on microscopic examination, NS colons revealed transmural inflammation involving all layers of the bowel wall with numerous granulocytes, monocytes, and lymphocytes. Conversely, the colons from high-dose simvastatin-treated TNBS mice were almost free of colitis. Inflammatory grade was also markedly improved in high-dose simvastatin-treated TNBS mice in comparison with NS mice (1.4 ± 0.3 , $n = 14$ vs. 2.9 ± 0.2 , $n = 17$; $P < 0.001$) (Fig. 3b).

We then investigated the effects of simvastatin after onset of TNBS-induced colitis (therapeutic protocol). No significant differences in body weight reduction were observed between simvastatin-treated (therapeutic protocol) and NS mice (Fig. 4; $12.9 \pm 2.3\%$, $n = 11$ vs. $12.8 \pm 2.0\%$, $n = 13$). With regard to inflammatory grade, the mean score in simvastatin-treated (therapeutic protocol) TNBS mice was significantly lower than that in NS mice significantly (Fig. 5; 2.3 ± 0.2 , $n = 11$ vs. 3.0 ± 0.2 , $n = 13$; $P < 0.05$).

Simvastatin suppresses Th1 cytokine production in TNBS colitis

The cytokine profile of T lymphocytes in TNBS-induced colitis was investigated by stimulating colonic LP cells

Fig. 3 Prophylactic administration of simvastatin improves the histologic lesions induced by TNBS.

(a) Microscopic appearance of colon (H&E stain; magnification 200 \times). CO—ethanol-treated mice. NS—TNBS-treated mice, showing severe transmural colitis. High—high-dose of simvastatin (prophylactic protocol). Note the marked reduction in colitis. (b) Inflammatory grade. Simvastatin dose-dependently improves the inflammation induced by TNBS. $**P < 0.001$ vs. NS

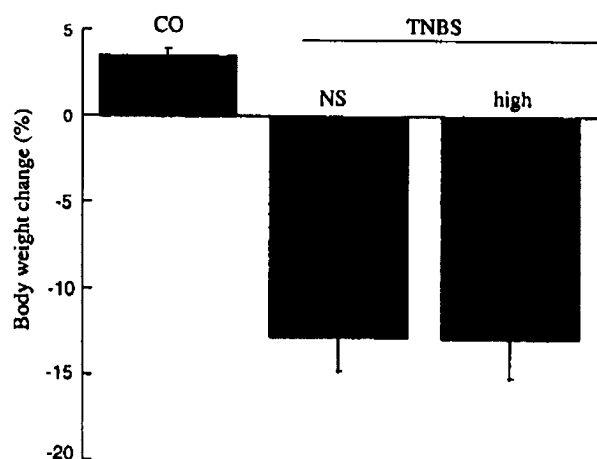
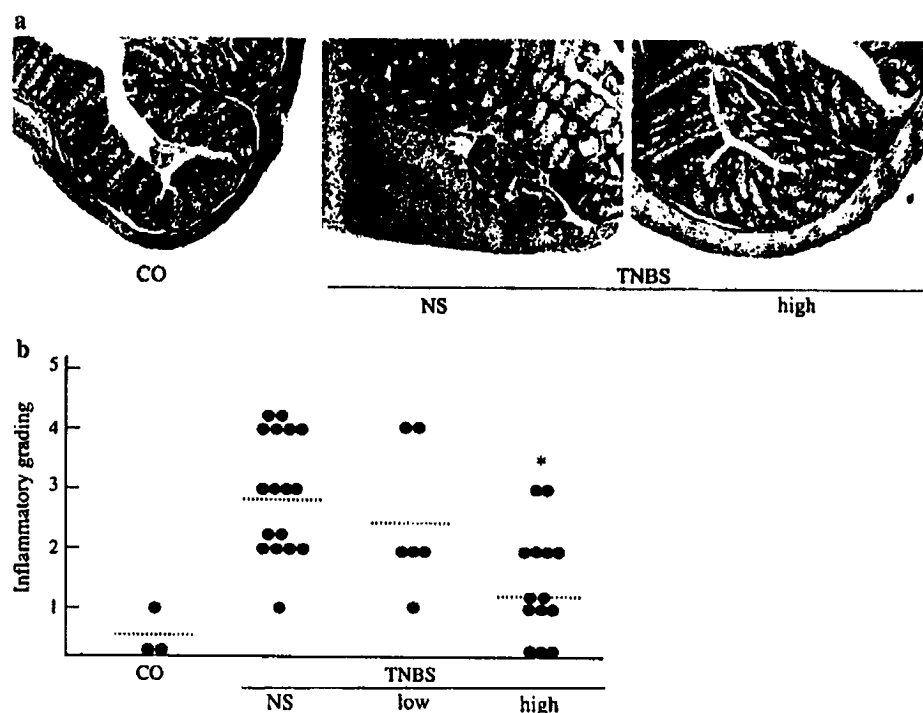


Fig. 4 Therapeutic administration of simvastatin shows no effect on body weight reduction. CO—ethanol-treated mice. NS—TNBS-treated mice. High—administration (therapeutic protocol) of high-dose simvastatin

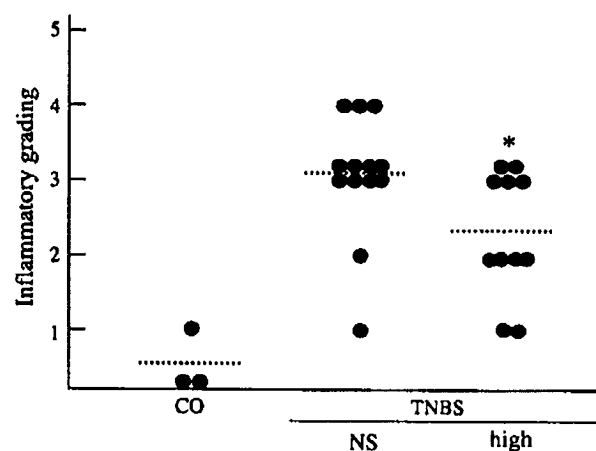


Fig. 5 Therapeutic administration of simvastatin improves histologic lesions induced by TNBS. CO—ethanol-treated mice. NS—TNBS-treated mice. High—administration (therapeutic protocol) of high-dose simvastatin. $*P < 0.05$

from NS mice via the antigen receptor (anti-TCR- β) and analyzing cytokine expression in the supernatants. As expected, colonic LP cells from NS mice produced large amounts of Th1 cytokines (IFN- γ and TNF- α) upon antigen-receptor stimulation (Fig. 6). In contrast, very little production of IL-4 or IL-5 was detected. Next, we investigated whether simvastatin (prophylactic protocol) suppresses the Th1-polarized immune response induced by

TNBS. IFN- γ release upon antigen-receptor stimulation was significantly suppressed in mice receiving high-dose simvastatin (simvastatin, 115.9 ± 36.6 pg/ml, $n = 8$ vs. NS, 436.8 ± 51.2 pg/ml, $n = 7$; $P < 0.001$). TNF- α release upon antigen-receptor stimulation was lower in mice receiving high-dose simvastatin than in NS mice, but this difference was not significant (193.2 ± 29.6 pg/ml, $n = 8$ vs. 312.9 ± 60.8 pg/ml, $n = 7$; $P = 0.11$).

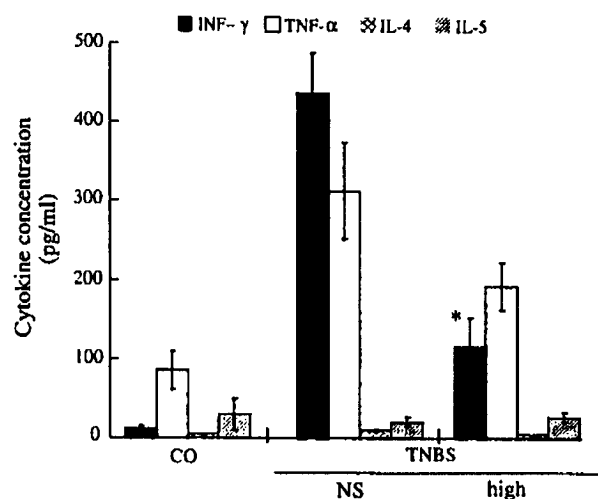


Fig. 6 Prophylactic administration of simvastatin suppresses Th1 cytokine production induced by TNBS. Colonic lamina propria cells were isolated and stimulated with anti-TCR- β and concentrations of INF- γ , TNF- α , IL-4, and IL-5 were measured in supernatants. INF- γ release was significantly suppressed in mice receiving high-dose simvastatin. * $P < 0.001$ vs. NS

Simvastatin did not prevent mice from developing OXA colitis

In order to determine whether simvastatin suppresses another immune-mediated colitis model, we investigated the effects of simvastatin on the development of OXA-induced colitis, which is caused by a polarized Th2-mediated response. In contrast to TNBS colitis, even high-dose simvastatin (prophylactic protocol) had no suppressive effects on either weight reduction (Fig. 7a; simvastatin, $13.3 \pm 1.5\%$, $n = 19$ vs. NS, $15.5 \pm 1.1\%$, $n = 20$; $P = 0.27$) or inflammatory grade (Fig. 7b; $2.8 \pm 0.2\%$, $n = 19$ vs. 3.2 ± 0.2 , $n = 20$; $P = 0.39$).

Discussion

In the present study, we provide experimental evidence that simvastatin negatively regulates the inflammatory response in TNBS-induced colitis. Moreover, we demonstrated that prophylactic administration is more effective than therapeutic administration in treating TNBS-induced colitis. TNBS-induced colitis is a hapten-induced model of human IBD. Intestinal inflammation in this model does not result from the direct effects of this chemical, but rather from delayed hypersensitivity to TNBS-modified self-antigens. The highly prophylactic effects of simvastatin on the development of TNBS-induced colitis suggest that statins may suppress not only acute inflammation, but also immune-mediated pathology.

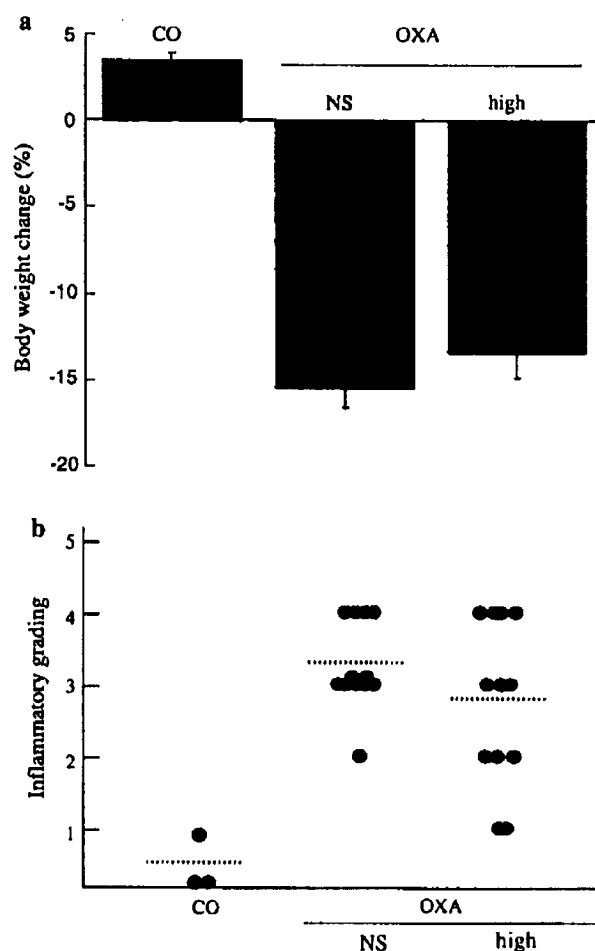


Fig. 7 Simvastatin shows no effect on body weight reduction (a) and histologic lesions (b) induced by OXA. CO—ethanol-treated mice. NS—OXA-treated mice. High—administration (prophylactic protocol) of high-dose simvastatin

Recently, special attention has been paid to the effect of statins on rodent colitis models. Sasaki et al. [13] reported that pravastatin relieved DSS-induced acute colitis in mice and promoted colonic endothelial nitric oxide synthase (eNOS) mRNA expression. That study also demonstrated that the protective effects of pravastatin were not present in eNOS-deficient mice. Similarly, Naito et al. [14] demonstrated that rosuvastatin suppresses intestinal inflammation and inhibits the increase in TNF- α , and the decrease in eNOS in DSS-induced acute colitis in mice. Lee et al. [15] showed that simvastatin inhibited TNF- α -induced IL-8 gene expression and nuclear transcription factor κ B (NF- κ B) transcriptional activity in intestinal epithelial cells. That study also showed that simvastatin prevents DSS-induced acute colitis in mice. Suzuki et al. [20] reported that fluvastatin inhibits colonic inflammation and incidence of colorectal dysplasia in DSS-induced chronic colitis in mice. Jahovic et al. [21] demonstrated that simvastatin and

fluvastatin reduce colonic inflammation and serum TNF- α levels in TNBS-induced colitis in rats. These results, taken together with those of the present study, suggest that statins are potential therapeutic agents for human IBD.

Several studies [22, 23] have demonstrated that statins can promote protective Th2 development and inhibit Th1 development in rodent models of Th1-mediated autoimmune disease. Liu et al. [22] demonstrated that atorvastatin ameliorates experimental autoimmune myocarditis, which is related to Th1 responses, accompanied by reductions in IFN- γ and IL-2 and the promotion of IL-4 and IL-10 production. Similarly, Aprahamian et al. [23] demonstrated that simvastatin reduces serum TNF- α and IFN- γ levels and increases IL-4 and IL-10 transcript levels in lymph nodes in a murine lupus model. In vitro, Arora et al. [24] demonstrated that simvastatin-treated murine dendritic cells promote T cells to secrete IL-4, IL-5, and IL-13, and this cytokine secretion profile was correlated with increased expression GATA-3 and decreased expression of T-bet by T cells.

Although CD and UC share clinical and pathological characteristics, each has a distinct cytokine production profile. CD is a Th1-mediated inflammatory disease [25, 26]. Various studies have shown predominant synthesis of IL-12 by macrophages and IFN- γ by T cells. In addition, mucosal lymphocytes in CD express high levels of active STAT-4, which is activated by IL-12 receptor signals and is necessary for the induction of Th1-associated genes. In this study, we used TNBS-induced colitis as a model of CD and demonstrated that simvastatin suppresses the Th1-polarized immune response induced by TNBS, similarly to other Th1-mediated autoimmune animal models.

On the other hand, based on various reports, such as the association between UC and the production of autoantibodies as well as the increased secretion of the Th2-related immunoglobulin subclasses IL-5 and IL-13, UC is believed to be a Th2-mediated inflammatory disease [25]. In this study, we used OXA-induced colitis as a model of UC, but simvastatin did not show any suppressive effects on either weight reduction or inflammatory grade in this model. In support of these results, there is a single case report of UC after pravastatin treatment [27]. The authors speculated that statins might reveal previously quiescent UC in susceptible individuals. There are several reports on other Th2-mediated autoimmune diseases. McKay et al. [28] demonstrated that simvastatin reduces IL-4 and IL-5 production in bronchoalveolar lavage fluid in a murine model of allergic asthma. In contrast, a randomized double-blind crossover trial [29] did not find any anti-inflammatory effects of simvastatin in patients with asthma. Based on the results regarding the effects of statins in promoting Th2-bias [22–24], as well as the present data, the use of statins

in Th2-mediated autoimmune disease must be evaluated cautiously. Further basic and clinical studies assessing the effects of statins on Th2-mediated autoimmune disease are thus necessary.

In conclusion, we demonstrated that simvastatin negatively regulates inflammation in TNBS-induced colitis, but not in OXA-induced colitis. In TNBS-induced colitis, simvastatin suppressed a Th1-polarized immune response. Our findings suggest that simvastatin has potential effects as a therapeutic agent in human IBD, particularly CD.

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